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# Regulatory T cells in skin lesions and blood of patients with bullous pemphigoid

Antiga E, Quaglini P, Volpi W, Pierini I, Del Bianco E, Bianchi B, Novelli M, Savoia P, Bernengo MG, Fabbri P, Caproni M

## Abstract

### Background

Although regulatory T cells (Tregs) are affected in several autoimmune skin diseases, only two studies have been performed in patients with bullous pemphigoid (BP) with contrasting results.

### Objective

To characterize Tregs and to determine the serum levels of regulatory cytokines in patients with BP.

### Methods

In BP lesional skin, immunohistochemistry and confocal microscopy were performed for CD4<sup>+</sup>, CD25<sup>+</sup>, forkhead/winged helix transcription factor (FOXP3)<sup>+</sup>, transforming growth factor (TGF)- $\beta$ <sup>+</sup> and interleukin (IL)-10<sup>+</sup> cells. In addition, the number of CD4<sup>+</sup>CD25<sup>++</sup>FOXP3<sup>+</sup> Tregs in peripheral blood was assessed by flow cytometry, and the levels of TGF- $\beta$  and IL-10 were determined in serum samples by enzyme-linked immunosorbent assay before and after steroid therapy. Controls included patients with psoriasis, atopic dermatitis (AD) and healthy donors.

### Results

The frequency of FOXP3<sup>+</sup> cells was significantly reduced in skin lesions from patients with BP ( $P < 0.001$ ) compared with psoriasis and AD. Moreover, the number of IL-10<sup>+</sup> cells was lower in BP than in psoriasis ( $P < 0.001$ ) and AD ( $P = 0.002$ ), while no differences were observed in the number of TGF- $\beta$ <sup>+</sup> cells. CD4<sup>+</sup>CD25<sup>++</sup>FOXP3<sup>+</sup> Treg in the peripheral blood of patients with BP was significantly reduced compared with healthy controls ( $P < 0.001$ ), and augmented significantly after steroid therapy ( $P = 0.001$ ). Finally, TGF- $\beta$  and IL-10 serum levels were similar in patients with BP compared with healthy controls. However, after therapy, BP patients showed significantly higher IL-10 serum levels than before therapy ( $P = 0.01$ ).

### Conclusions

These data suggest that the depletion of Tregs and of IL-10 in patients with BP may be an important factor in the pathogenesis of the disease.

## Introduction

Regulatory T cells (Tregs) represent a subset of immune effector cells, firstly described by Sakaguchi *et al.*, [1] which play a central role in immune homeostasis by regulating the maintenance of self-tolerance and are therefore essential for the control of immune responses. [2-4]

Currently, distinct subsets of Tregs have been described,[2] subdivided on the basis of different expression of cell surface markers, production of cytokines and mechanism of action.[5] Naturally occurring thymic-derived CD4<sup>+</sup>CD25<sup>+</sup> Tregs (nTregs) represent 1–10% of total CD4<sup>+</sup> T cells in thymus, peripheral blood and lymphoid tissues, and acquire their regulatory capacity during normal thymopoiesis. Phenotypically, nTregs are characterized by the bright expression of CD25 and by the constitutive expression of specific markers, such as the transcription factor forkhead box P3 (FOXP3),[4] the glucocorticoid-induced tumour-necrosis factor receptor-related protein and the cytotoxic T-lymphocyte associated antigen 4. Another group of CD4<sup>+</sup>CD25<sup>+</sup> Tregs is represented by the ‘antigen induced or adaptive’ Tregs (aTregs). The suppressive function of these induced Tregs is mediated by the production of the suppressive cytokines interleukin (IL)-10 and transforming growth factor (TGF)- $\beta$ .

Recent literature proposed that a lack of Tregs number or a defect in function could potentially lead to loss of tolerance and thus to the development of autoimmunity. Indeed, numerous autoimmune and immune-mediated diseases (including gastritis, thyroiditis, type 1 diabetes, myasthenia gravis, rheumatoid arthritis and multiple sclerosis) have been shown to present significant depletion in the number and/or function impairment of Tregs.[2-4, 6, 7] A significant down-modulation of Tregs was found also in different immune-mediated dermatoses, such as psoriasis, scleroderma, dermatomyositis, lupus erythematosus and graft-versus-host disease.[8-12]

To date, only a few studies focused on Tregs in autoimmune bullous skin diseases, a group including different clinical-pathological entities characterized by the development of autoimmunity against structural components maintaining cell-cell (pemphigus group) and cell-matrix [bullous pemphigoid (BP) group] adhesion. In particular, BP, the most common autoimmune blistering disease, is characterized by the development of auto-antibodies directed against components of the hemidesmosome adhesion complex, the BP230 and collagen XVII/BP180 proteins. Although the underlying mechanisms involved in triggering the autoimmune response in BP are not well-understood, BP is now considered a T-cell dependent autoimmune disease with the presence of CD4<sup>+</sup> autoreactive T cells that recognize the ectodomain of BP180.[13-15] The pathogenesis of the disease involves also other factors, including complement activation, mast cell degranulation, accumulation of inflammatory cells (eosinophils, neutrophils) and the release of proteases.[13, 16-18]

Although Tregs pathway seems to be impaired in pemphigus vulgaris,[19-22] contrasting results are reported for BP. In fact, while Rensing-Ehl *et al.* did not find differences between BP patients and healthy controls,[23] more recently a study by Arakawa and co-workers[24] and a study from our research group[25] demonstrated a reduction in Tregs numbers in the skin and in the blood of BP patients respectively.

In this study, to confirm our previous results and to analyse further the role of Tregs in the pathogenesis of BP, the expression and distribution of both naturally-occurring FOXP3<sup>+</sup> Tregs and ‘antigen-induced’ IL-10 and TGF- $\beta$  producing Tregs, have been investigated using immunohistochemistry, confocal microscopy, flow-cytometry and ELISA both in the lesional skin and in the blood of patients with BP.

## Materials and methods

### Patients

A total of 21 patients with BP (9 M, 12 F; median age 68 years, range 39–84) were included into the study. The diagnosis of BP was made on the basis of a typical clinical presentation of classic BP

with diffuse blistering of the skin, subepidermal blistering with granulocytic infiltrate at the histopathology, linear immunoglobulin G (IgG) and complement three deposits along the basement membrane zone at the direct immunofluorescence examination, linear IgG deposits at the blister roof of sodium chloride split human skin at the indirect immunofluorescence, and presence of circulating anti-BP180 antibodies by ELISA.

Skin biopsy specimens and blood samples from patients with psoriasis ( $n = 10$ , 4 M, 6 F; median age 44 years, range 26–61) and atopic dermatitis (AD) ( $n = 10$ ; 5 M, 5 F, median age 38 years, range 22–59) were used as controls, along with skin and blood samples from 30 age- and gender-matched healthy controls (HC). The patients and controls were recruited from two Italian Dermatologic Clinics (Florence and Turin) belonging to the Italian Group for Cutaneous Immunopathology.

All patients and controls underwent a skin biopsy and a peripheral blood test during the first visit and before any topical and/or systemic treatment was started. Furthermore, a blood sample was collected from 11 BP patients at the end of consolidation phase as defined by Murrell *et al.*[26] Table 1 shows the therapy regimens and the time needed to achieve the end of consolidation phase for each BP patient included into the study.

Table 1. Therapy regimens and time to achieve end of consolidation phase for each patient

<b>Patient no</b>	<b>Therapy (higher dosage)</b>	<b>Time to achieve end of consolidation phase</b>
1	Prednisone 0.5 mg/Kg/day	3 months
2	Topical clobetasol 20 g/day	2 months
3	Prednisone 0.75 mg/Kg/day	4 months
4	Prednisone 0.5 mg/Kg/day	3 months
5	Prednisone 0.5 mg/Kg/day	2 months
6	Prednisone 0.75 mg/Kg/day	3 months
7	Prednisone 0.5 mg/Kg/day + azathioprine 200 mg/day	5 months
8	Topical clobetasol 25 g/day	45 days
9	Prednisone 1 mg/Kg/day	3 months
10	Prednisone 0.5 mg/Kg/day + azathioprine 200 mg/day	3 months
11	Prednisone 0.5 mg/Kg/day	45 days
12	Prednisone 0.5 mg/Kg/day	4 months
13	Prednisone 1 mg/Kg/day	5 months
14	Prednisone 0.75 mg/Kg/day	3 months
15	Topical clobetasol 20 g/day	3 months
16	Prednisone 0.5 mg/Kg/day + azathioprine 200 mg/day	4 months
17	Prednisone 1 mg/Kg/day	3 months
18	Topical clobetasol 30 g/day	3 months
19	Topical clobetasol 25 g/day	2 months
20	Prednisone 0.5 mg/Kg/day	45 days
21	Prednisone 1 mg/Kg/day	2 months

The trial was approved by the medical ethical committee of each Hospital and was conducted according to the Declaration of Helsinki. All the patients and controls provided written informed consent to participate to the study.

## **Immunohistochemistry**

The expression of different Tregs markers was analysed in serial sections of lesional skin biopsy specimens of 15 BP patients, 10 psoriasis patients, 10 AD patients, as well as in healthy skin of eight HC using single immunohistochemical staining as described previously.[27]

Skin specimens, that had been immediately frozen at  $-80^{\circ}\text{C}$  in liquid nitrogen, were cut into 5- $\mu\text{m}$  thick sections and stained immunohistochemically. The monoclonal antibodies included those to CD4 (1 : 50; DAKO, Copenhagen, Denmark), CD25 (1 : 10; DAKO), FOXP3 (1 : 40; Abcam Ltd., Cambridge, UK), TGF- $\beta$  (1 : 2000; R&D Systems, Minneapolis, MN, USA) and IL-10 (1 : 50; DAKO). Before staining, frozen sections were air-dried and fixed in acetone (5 min).

Immunolabeling was performed with the alkaline phosphatase/antialkaline phosphatase complexes (1 : 30 dilution, 30 min; DAKO). FOXP3 peptide ab14151 (0.125 mg/mL; Abcam) was used as a control to block anti-FOXP3 binding.

Two independent 'blind' observers evaluated the slides. For quantitative analysis, the stained cells were counted in three consecutive microscopic fields ( $400\times$ ). Furthermore, for each patient, FOXP3<sup>+</sup>/CD4<sup>+</sup>, TGF- $\beta$ <sup>+</sup>/CD4<sup>+</sup> and IL-10<sup>+</sup>/CD4<sup>+</sup> cell ratios were calculated by counting the absolute number of positively stained cells in serial sections from the same skin biopsy specimen.

## **Confocal microscopy**

About 10- $\mu\text{m}$  serial sections of fresh frozen skin biopsies from eight patients with BP, five with psoriasis, five with AD and five HC were fixed with ice cold acetone then blocked with 0.25% casein in Tris-buffered saline (TBS; 0.88% NaCl, ThermoFisher, 0.24% tris(hydroxymethyl)aminomethane, (VWR International) and distilled water). Primary antibodies against human CD4 (mouse IgG2b, OKT4, 1 : 50, BioLegend, San Diego, CA, USA) and FOXP3 (mouse IgG1, 206D, 1 : 50, BioLegend) were prepared in TBS containing 1% FCS and incubated on sections for 1 h at room temperature. Secondary antibodies against mouse isotypes IgG2a DyLight 488 (poly24092, BioLegend) and IgG2b AlexaFluor 555 (Molecular Probes, Grand Island, NY, USA) at 1 : 200 each were prepared and incubated on sections for 1 h. Sections were visualized with Nikon C2 confocal microscope (Nikon, Tokyo, Japan).

## **Flow cytometric analysis**

The flow cytometric analysis was performed at a single site (Turin). Peripheral blood lymphocyte samples from 21 BP patients before therapy and 11 BP patients (number 1, 3, 4, 5, 6, 7, 8, 12, 13, 17, 19; see Table 1) after therapy were evaluated according to their immunofluorescence reactivity, using FACSCalibur or FACSCanto™ II cytometer and analysed with CellQuest or FACSDiva software (Becton Dickinson, San Jose, CA, USA). Surface markers on blood were performed by four or six-colour immunofluorescence analyses, using simultaneously antibodies conjugated to FITC, PE, PerCP or PerCP Cy5.5, Pe-Cy7, allophycocyanin (APC) and APC-H7. At least 10 000 lymphocytes were collected for each antibody combination. Lymphocyte purity was verified by the standard forward and sideways scattering parameters, by means of a CD45 gating analysis. A wide panel of mAbs directed against T-cell antigens was routinely tested to screen patients' phenotypes. The following mAbs were analysed for this study: anti-CD4 PerCP or Pe-Cy7 (clone SK3, mouse IgG1) from (BD Biosciences, San Jose, CA, USA) anti-CD25 PE, or APC (clone CD25-3G10,

mouse IgG1) from Caltag, Burlingame CA, USA). The CD4<sup>+</sup>CD25<sup>+</sup> population can be divided into two different levels of expression, i.e. cells that express a low CD25 level (defined CD4<sup>+</sup>CD25<sup>low</sup>) and cells with a higher CD25 level (defined CD4<sup>+</sup>CD25<sup>bright</sup>) that appear to have a tail to the right of the major population containing both CD4<sup>+</sup>CD25<sup>low</sup> and CD4<sup>+</sup>CD25<sup>+</sup> cells. FOXP3 cytometric expression was analysed using mononuclear cells purified from peripheral blood using Lymphoprep (1.077 g/mL; Axis-Shield Poc As, Oslo, Norway) density gradient centrifugation. Firstly, the cells were incubated with surface antibodies, anti-CD4 PerCP or PE-Cy7 and anti-CD25 APC for 10 min at room temperature. They were then stained with anti-FOXP3 PE (clone PCH101, rat IgG2a; eBiosciences, San Diego, CA, USA), according to the manufacturer's protocol, using fixation and permeabilization buffers from the same provider. Evaluation of Tregs subpopulations by flow cytometry was determined as the percentage expression of CD25<sup>bright</sup>FOXP3<sup>+</sup> within the CD3<sup>+</sup>CD4<sup>+</sup> compartment. Blood samples of 10 patients with psoriasis, 10 with AD and 30 HC were investigated as controls; data of the patients with psoriasis and AD were retrieved from our previous study, where the same methods were used.[10]

### Enzyme-linked immunosorbent assay

As for flow cytometric assay, even the ELISA was performed at a single site (Florence). Blood samples from 21 BP patients were collected at the diagnosis and after the resolution of the disease and were centrifuged for 20 min at 10000 g (5417R microcentrifuge; Eppendorf, Hamburg, Germany). Then, serum samples were subdivided into small aliquots to be stored at -80°C until tested for cytokine levels. Solid phase ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to determine TGF-β1 and IL-10 serum levels, according to the manufacturers' instructions. Samples and standards were analysed in duplicates and only variation coefficients <15% were accepted. As control, serum samples of 10 HC were analysed.

### Statistical analysis

The results are presented as medians [25th–75th percentile]. The Mann–Whitney *U*-test was used to compare the number of Tregs in peripheral blood of patients and healthy controls and the results of the immunohistochemical and confocal microscopy analysis. Both the Kruskal–Wallis with Dunn *post hoc* test and the Student's *t*-test were used to compare the data from the ELISA analysis, giving similar results. The results of flow cytometric analysis and ELISA in BP patients before and after treatment were paired and analysed using Wilcoxon signed rank test. The correlation between the percentages of circulating Tregs with IL-10 serum levels in BP patients after treatment was calculated by Spearman's test. Results were considered significant with a *P* value <0.05.

## Results

### CD4<sup>+</sup>, CD25<sup>+</sup> and FOXP3<sup>+</sup> nTregs in the skin of patients with BP (Table 2)

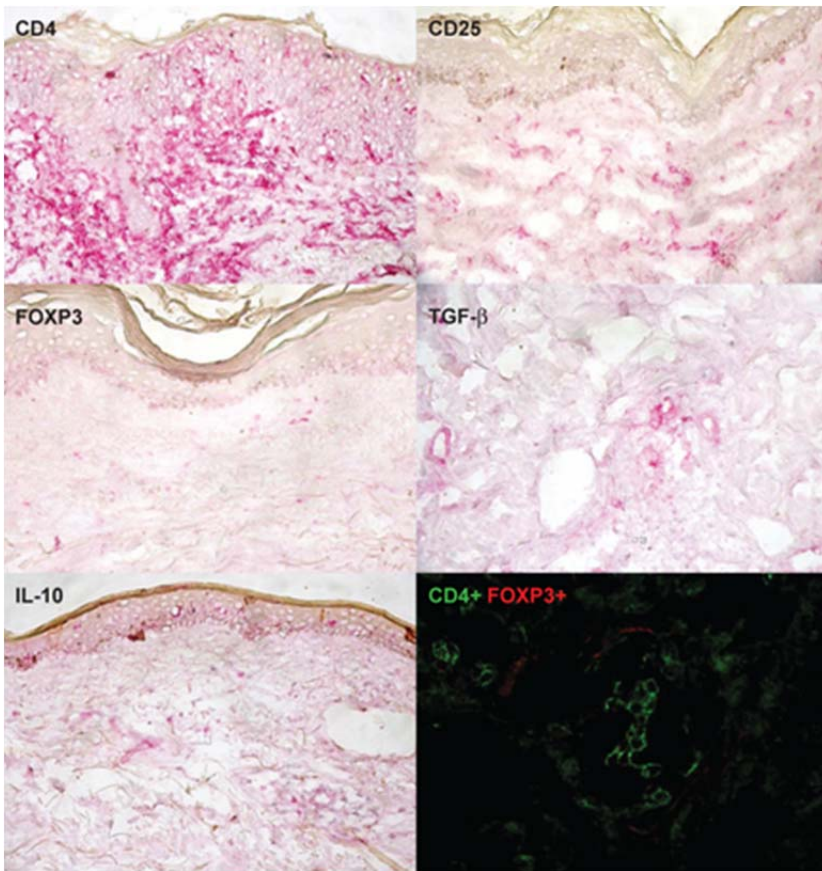
CD4<sup>+</sup> and CD25<sup>+</sup> cells were located in the superficial dermis in patients with BP (Fig. 1).

Table 2. Quantitative analysis on the numbers of positive cells for field (400X) in skin lesions of bullous pemphigoid patients and controls as assessed by immunohistochemistry and confocal microscopy

	BP	HC	Psoriasis	AD
1. Positive-cell counts are expressed as medians [25th–75th percentile].				
2. BP, bullous pemphigoid; HC, healthy controls; AD, atopic dermatitis.				
CD4 <sup>+</sup> cells	61 [53.5–67.5]	5 [4–6.5]	71.5 [57.3–77.3]	61.5 [54–72.7]
CD25 <sup>+</sup> cells	17 [5.5–25.5]	2 [1–3]	35 [26.7–42.7]	33 [27.5–42.7]
FOXP3 <sup>+</sup> cells	1 [0–2]	1 [0–1.2]	27.5 [20–32.5]	22.5 [16.7–26.5]
% FOXP3 <sup>+</sup> /CD4 <sup>+</sup>	1.9 [0–3.1]	18.7 [0–25]	33.7 [31.1–39.5]	37.6 [31.3–41.4]
CD4 <sup>+</sup> FOXP3 <sup>+</sup> cells	0.5 [0–2]	0 [0–1]	22 [17.5–28]	19.5 [16–25]
TGF-β <sup>+</sup> cells	20 [15.5–27]	2.5 [1.7–3]	22.5 [12.5–27.7]	19.5 [12.5–23.7]
% TGF-β <sup>+</sup> /CD4 <sup>+</sup>	42.2 [22.9–51.1]	38.7 [32.3–48.7]	34.2 [19.3–39]	35.6 [21.4–41.6]
IL-10 <sup>+</sup> cells	1 [0–2.5]	1 [1–1.2]	16.5 [9.2–22]	5.5 [4–7.7]
% IL-10 <sup>+</sup> /CD4 <sup>+</sup>	1.6 [0–4]	25 [18.1–25]	24.6 [15.5–31.2]	9.5 [5.7–15.3]



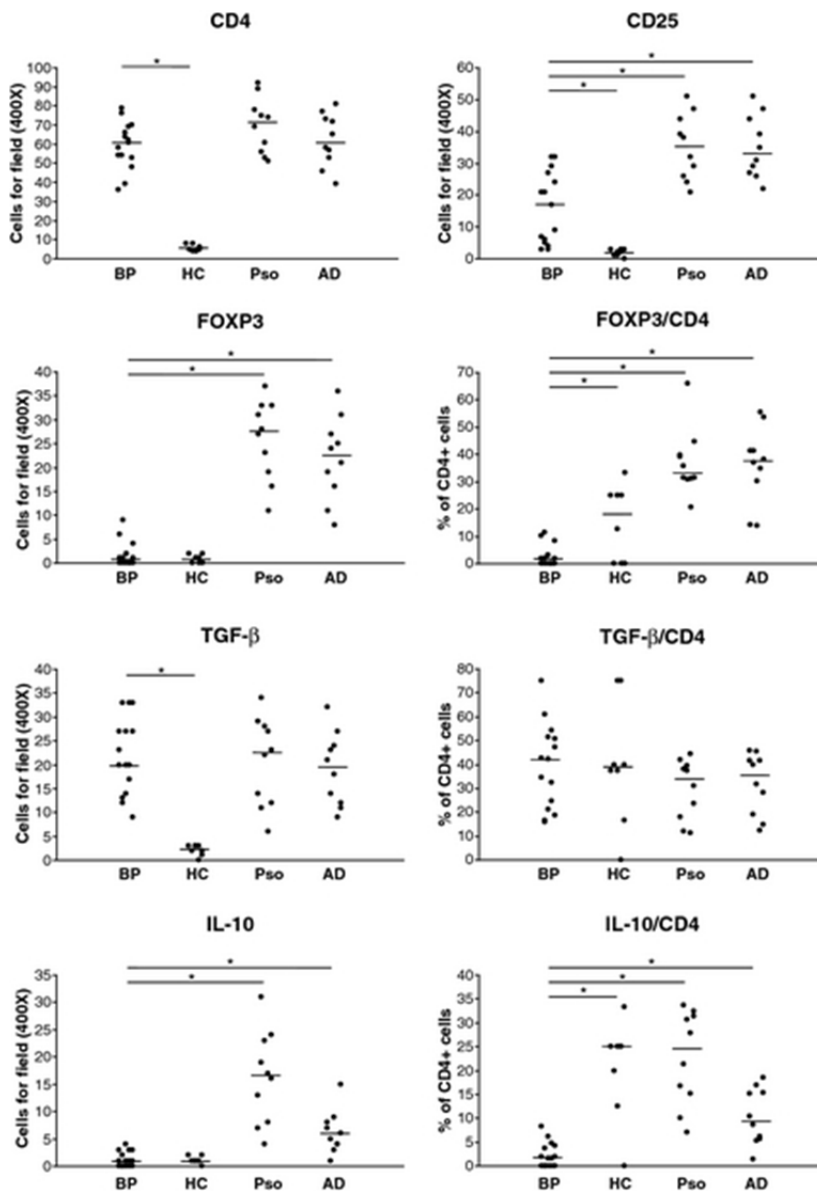
**Figure 1.**



Immunohistochemical staining for CD4, CD25, FOXP3, TGF- $\beta$  and IL-10, as well as double staining of CD4<sup>+</sup>FOXP3<sup>+</sup> cells assessed by confocal microscopy, in skin biopsy specimens from patients with bullous pemphigoid. Positive cells are mainly distributed within the superficial dermis.

The level of CD4<sup>+</sup> cell infiltration was similar in patients with BP, psoriasis and AD. HC showed significantly lower numbers of CD4<sup>+</sup> infiltrating cells than BP and control diseases (HC vs. BP, psoriasis and AD:  $P < 0.001$ ) (Fig. 1). In contrast, the frequency of CD25<sup>+</sup> cells was significantly lower in BP skin lesions compared with psoriasis ( $P = 0.008$ ) and AD ( $P = 0.01$ ), and higher compared with HC ( $P < 0.001$ ) (Fig. 2).

**Figure 2.**



Numbers of CD4<sup>+</sup>, CD25<sup>+</sup>, FOXP3<sup>+</sup>, TGF-β<sup>+</sup> and IL-10<sup>+</sup> cells as well as FOXP3/CD4, TGF-β/CD4 and IL-10/CD4 cell ratios in the inflammatory infiltrate of skin biopsy specimens from patients with bullous pemphigoid, healthy controls or patients with psoriasis and atopic dermatitis. BP, bullous pemphigoid; HC, healthy controls; PSO, psoriasis; AD, atopic dermatitis; \**P* < 0.05.

In BP inflammatory infiltrate, few FOXP3<sup>+</sup> cells were identified within the superficial dermis (Fig. 1). In contrast, patients with psoriasis and AD revealed a significantly higher number of FOXP3<sup>+</sup> cells in the infiltrate (BP vs. psoriasis: *P* < 0.001; BP vs. AD: *P* = 0.001) (Fig. 2). No differences could be found in the absolute number of FOXP3-expressing cells between BP patients and HC (Fig. 2).

The binding of FOXP3 antibody could be blocked by preincubating the antibody with specific FOXP3 peptide in skin specimens. For control purposes, an isotype-matched control antibody was used and consistently yielded negative results. The frequency of FOXP3<sup>+</sup> cells, expressed as the percentage of CD4<sup>+</sup> cells, was found to be significantly lower in BP specimens compared with HC (*P* = 0.009), psoriasis (*P* < 0.001) and AD (*P* < 0.001) (Fig. 2).

Finally, the number of CD4<sup>+</sup>FOXP3<sup>+</sup> double stained cells investigated by confocal microscopy (Fig. 1) was significantly lower in BP patients and in HC than in patients with psoriasis and AD (BP vs. psoriasis:  $P < 0.001$ ; BP vs. AD:  $P = 0.001$ ).

### Quantification of TGF- $\beta$ <sup>+</sup> and IL-10<sup>+</sup> cells in the skin of patients with BP (Table 2)

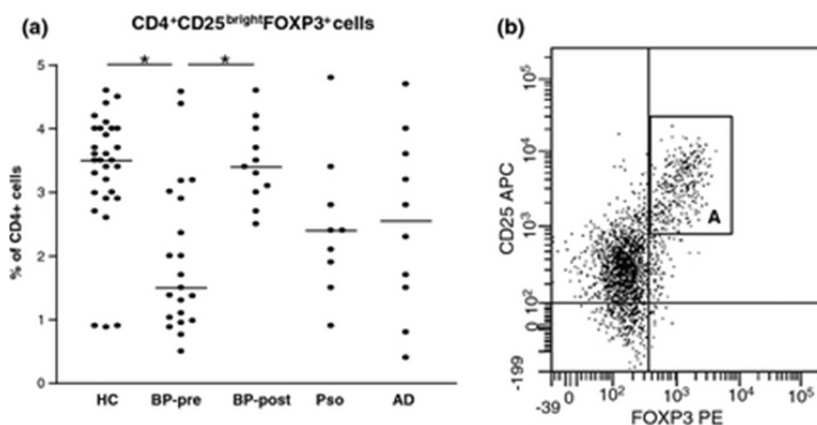
Several TGF- $\beta$ <sup>+</sup> cells preferentially located in the superficial dermis (Fig. 1) were detected in BP, without statistical differences compared with psoriasis and AD (Fig. 2). In contrast, HC showed a reduced number of TGF- $\beta$ <sup>+</sup> if compared with BP ( $P < 0.001$ ), psoriasis ( $P < 0.001$ ) and AD ( $P < 0.001$ ). Finally, TGF- $\beta$ <sup>+</sup>/CD4<sup>+</sup> cell ratio was similar in BP, HC, psoriasis and AD skin specimens.

Only few IL-10<sup>+</sup> cells were found in the superficial dermis of BP skin lesions (Fig. 1). Their number was similar to that found in HC, but significantly lower than in patients with psoriasis ( $P < 0.001$ ) or AD ( $P = 0.02$ ) (Fig. 2). Furthermore, IL-10<sup>+</sup>/CD4<sup>+</sup> cell ratio was lower in BP skin lesions than in HC ( $P < 0.001$ ), in AD ( $P = 0.003$ ) and in psoriasis ( $P < 0.001$ ).

### Peripheral blood Tregs quantification (Figure 3)

The presence of CD25<sup>bright</sup>FOXP3<sup>+</sup> within the CD4<sup>+</sup> circulating compartment was analysed in BP and compared with HC, psoriasis and AD patients (Fig. 3a). The dot plot of a representative BP patient is reported in Fig. 3b.

**Figure 3.**



(a) Percentages of the CD25<sup>bright</sup>FOXP3<sup>+</sup> subpopulation within the CD4<sup>+</sup> cell subset in bullous pemphigoid patients (before and after therapy) compared with healthy controls and patients with psoriasis and atopic dermatitis. Each dot represents a patient; horizontal bars indicate median values. HC, healthy controls; BP-pre, bullous pemphigoid before therapy; BP-post, bullous pemphigoid after therapy; PSO, psoriasis; AD, atopic dermatitis; \* $P < 0.05$ . (b) Representative dot plot from a BP patient. The gate A shows CD25<sup>bright</sup>FOXP3<sup>+</sup> subpopulation within the CD4<sup>+</sup> cell subset.

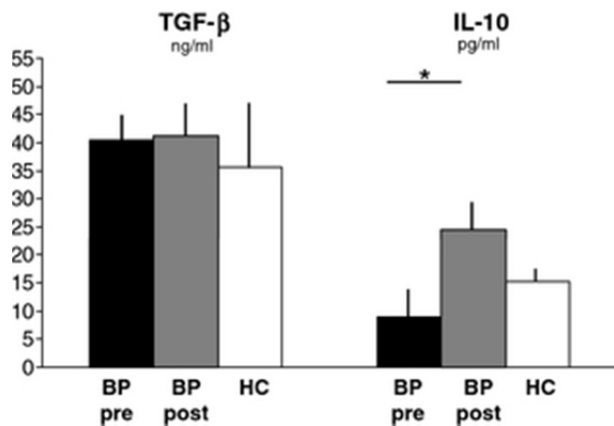
The frequency of CD25<sup>bright</sup>FOXP3<sup>+</sup> within the CD4<sup>+</sup> subset was significantly reduced in BP patients (median [25th–75th percentile]: 1.5%[1%–2.9%]) with respect to HC (median [25th–75th percentile]: 3.5%[2.9%–4%];  $P < 0.001$ ). The percentages of the CD25<sup>bright</sup>FOXP3<sup>+</sup> subset in BP patients were also slightly lower than those of patients with both psoriasis and AD (median: 2.5% and 2.6% respectively), even if this difference was not significant ( $P = 0.07$ ).

The analysis of the circulating Tregs kinetics after treatment was available only in 11 patients. Interestingly, in these cases, the resolution of the skin lesions was associated with a parallel increase in the percentages of the CD25<sup>bright</sup>FOXP3<sup>+</sup> cell subset (median [25th–75th percentile]: 3.4%[2.5–4];  $P = 0.001$  with respect to baseline) up to HC values.

### TGF- $\beta$ and IL-10 concentrations in sera of BP patients before and after therapy

To assess the concentrations of the regulatory cytokines TGF- $\beta$  and IL-10, serum samples were investigated both in patients with BP and in HC. TGF- $\beta$  concentrations were similar both in BP patients before treatment (median [25th–75th percentile]: 40.4 ng/mL [31.1–44.4]) and in HC (median [25th–75th percentile]: 35.5 ng/mL [33.7–45.3]). Moreover, TGF- $\beta$  serum levels were not affected by therapy, and remained almost unchanged in treated patients after the resolution of the disease (median [25th–75th percentile]: 41.1 ng/mL [33.6–44.9]) (Fig. 4).

**Figure 4.**



Serum levels of the regulatory cytokines TGF- $\beta$  and IL-10 in patients with bullous pemphigoid before and after therapy and in healthy controls. BP-pre, bullous pemphigoid before therapy; BP-post, bullous pemphigoid after therapy; HC, healthy controls; \* $P < 0.05$ .

No significant differences in IL-10 concentrations were found between patients with BP before treatment (median [25th–75th percentile]: 8.9 pg/mL [4.3–15.7]) and HC (median [25th–75th percentile]: 15.2 pg/mL [14.4–17.5]). However, after therapy and thus after the resolution of the disease, BP patients showed significantly higher IL-10 serum levels than before therapy (median [25th–75th percentile]: 24.5 pg/mL [17.7–31.2];  $P = 0.01$ ) (Fig. 4). Interestingly, in treated patients, IL-10 serum levels showed a statistically significant correlation with the percentage of CD4<sup>+</sup>CD25<sup>bright</sup>FOXP3<sup>+</sup> cells ( $P = 0.02$ ).

## Discussion

This study was designed to characterize the expression of both naturally-occurring and antigen-induced Tregs in skin biopsies and blood samples from patients with BP, to evaluate their potential role in the pathogenesis of the disease. Our main finding was the demonstration of a significant reduction in Tregs in patients with BP compared with HC.

Interestingly, our data showed that the decreased frequency of FOXP3<sup>+</sup> Tregs was simultaneously present both in the skin and peripheral blood of patients with BP. According to a recent study by our research group,[25] this finding suggests that the reduction in Tregs observed in the blood was not the result of a preferential homing of these cells into the skin, or *vice versa*, but rather it reflects their real downregulation, and therefore, their potential impaired suppressive function that could play a role in the pathogenesis of the disease.

Our results are in contrast with those by Rensing-Ehl *et al.*,[23] who did not find differences in the number of circulating Tregs between BP patients and HC. In their study, however, the analysis of circulating Tregs was made only the basis of the CD25<sup>bright</sup> expression, while no specific data on FOXP3<sup>+</sup> cells in BP and HC were shown. Therefore, in that article, CD4<sup>+</sup>CD25<sup>bright</sup> cells could probably represent not only Tregs but also FOXP3<sup>-</sup> activated T cells, that are increased in BP. Moreover, Rensing-Ehl *et al.* found FOXP3<sup>+</sup> Tregs also in the lesional skin of patients with BP[23]; however, they did not perform any cell count or statistical analysis, and did not calculate the FOXP3<sup>+</sup>/CD4<sup>+</sup> ratio. The latter, rather than the absolute number of Tregs, is considered an important parameter in the evaluation of Tregs immunosuppressive activity, as Tregs/T effector cell ratios are decreased in patients with autoimmune and inflammatory diseases.[10, 11, 28] Accordingly, very recently Arakawa and coworkers found a reduced FOXP3<sup>+</sup>/CD4<sup>+</sup> ratio in lesional skin of BP patients than in patients with pemphigus.[24]

Interestingly, after a steroid course and thus after the end of consolidation phase of BP, we detected an increase in the number of circulating CD25<sup>bright</sup>FOXP3<sup>+</sup> cells up to the values found in HC. This finding is in agreement with previous data from the literature, showing that immunosuppressive therapy either with oral steroids or with other immunosuppressant drugs is able to increase the pool of circulating Tregs.[29, 30] Although we tested only 11 patients after therapy and thus our results need further confirmation, the activity on Tregs could potentially be considered one of the curative effects of steroids in patients with BP.

In this study, we also investigated TGF- $\beta$  and IL-10, regulatory cytokines involved in Tregs suppressive function and particularly expressed by aTregs. Interestingly, while no differences were found in TGF- $\beta$ <sup>+</sup>/CD4<sup>+</sup> T cell ratio in BP patients and controls, the percentages of IL-10<sup>+</sup> cells among CD4<sup>+</sup> cells were significantly reduced in skin lesion of BP, suggesting a local deficiency of such cells, that could lead to a defective regulatory function.

In contrast, we did not find any significant differences in the serum levels of both TGF- $\beta$  and IL-10 between patients with BP and HC. In agreement with our data, several studies from the literature demonstrated that, while IL-10 seems to be elevated in the blister fluid of patients with BP, its serum concentration overlaps that of healthy subjects.[31-33]

However, after the resolution of the disease, BP patients showed higher IL-10 serum levels than before treatment. These findings might imply a relative lack of IL-10 in BP patients, where the levels of this cytokine, overlapping those of HC, could not be able to ensure an adequate immunoregulation in a state of enhanced reactivity such as BP. After steroid therapy which, as discussed above, is able to enhance Tregs numbers and function, IL-10 concentrations augmented consequently and reached levels that could be able to balance the autoimmune response present in BP patients. Accordingly, Teraki *et al.* demonstrated that corticosteroids may control inflammatory responses in BP by promoting expansion of IL-10-producing cells.[34] Interestingly, we also found a positive correlation between IL-10 serum levels and circulating Treg percentages in BP patients after treatment, suggesting that they are strictly related.

IL-10 is a potent antiinflammatory cytokine that is able to suppress production of both type 1 and type 2 cytokines.[35] A number of studies have shown beneficial effects of IL-10 in different models of cell-mediated autoimmunity,[36, 37] including a model of BP.[38] Moreover, IL-10 is able to potentiate the IL-4-induced synthesis of IgG4,[39] a IgG subclass that is less pathogenic than IgG1 in patients with BP, as it is not capable of fixing complement.[40] Thus, taken as a whole, these data suggest that IL-10 could represent a therapeutic approach for the management of BP.[34]

Our study, however, has some limitations, including the fact that FOXP3, although still considered the most specific marker for Tregs, can be transiently expressed on activated T cells. Further studies including functional assays and the use of other markers for Tregs, such as CD127<sup>low</sup> and CD39<sup>high</sup>,[41] would augment the specificity of the results.

To conclude, despite such limitations, our findings suggested a reduction in both skin-homing and circulating FOXP3<sup>+</sup> Tregs in patients with BP, together with a downregulation of the IL-10 pathway. The resolution of the disease after a course with systemic corticosteroids was paralleled by an increase in circulating Tregs and of IL-10 serum levels, suggesting a possible alternative way of action of these drugs other than affecting T effector cells.

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